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Leigh Erin Fuller
University of North Florida

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Leigh Erin Fuller

Faculty Sponsor: John D. Hatle,
Assistant Professor of Biology

Abstract

Diet is the only environmental variable that has been shown to slow aging. Late-onset caloric restriction results in increased longevity in female grasshoppers. To better understand the physiological processes involved, and how rapid the responses can occur, we studied the effect of diet on total fat body protein. We tested the effects of short-term treatments on the amount of fat body protein for day 20 and day 50 of adulthood: *ad libitum* feeding (free access to food), starvation (only water), caloric restriction (1.5 g of lettuce) and heat shocking (60 min at 48°C). Grasshoppers were initially fed *ad libitum* and then switched to diet treatments 48 h before fat body removal. We found that day 20 grasshoppers had more fat body protein than day 50 grasshoppers. At day 20, but not at day 50, *ad libitum* feeding produced higher levels of fat body protein than other diets. Our results suggest that total fat body protein is not one of the physiological responses that produce longer lifespan when organisms are on a caloric restriction diet.

Introduction

Aging refers to the biological changes that occur during a lifetime that result in reduced resistance to stress, increased vulnerability to disease and injury, reduced probability of healthy reproduction (in females) and an increased probability of death (1). The rate of aging is species specific, suggesting a strong genetic influence. The only environmental variable that has been shown to affect the rate of aging in a wide range of species is diet (2).

Specifically, calorie restriction, consuming fewer calories while acquiring sufficient nutrients, extends lifespan by 30-100%, depending on the organism (1). Cao and colleagues (3) found that mice on a short-term caloric restriction diet after 4 weeks had reversed most effects of aging in liver cells, which are changes in gene expression associated with increased inflammation, cellular stress, and fibrosis, and reduced capacity for apoptosis, xenobiotic metabolism, normal cell-cycling, and DNA replication. Another study found that late-onset calorie restriction in mice starting at 14 months of age resulted in a 19% global inhibition of age-related changes in gene expression (4).

Another important study by Mair and colleagues (5) found that late-onset caloric restriction in flies decreased the risk of mortality in comparison to flies on full-fed diet. They found that when fully fed flies where switched to a calorie restriction diet the mortality rate decreased within 48 hours to match the level of mortality in flies that were on a calorie restriction diet their entire adult life. Also, when the reverse was performed, switching flies from a calorie restriction diet to a full-fed diet, their mortality rate rapidly increased, within 48 hours. These were important findings because prior to this it was believed that caloric restriction did not have rapid benefits, instead an organism had to be on a calorie restriction diet their entire life.

In a previous study we tested the effect of diet on female lubber grasshopper longevity. There were 4 treatment groups in this study: *ad libitum* (free access to food), 70% caloric restriction (offered 70% of what the *ad libitums* ate), 60% caloric restriction (offered 60% of what the *ad libitums* ate) and late-onset caloric restriction (offered free access to food the first 50 days of adulthood and then switched to 60% calorie restriction diet until death). The median death in days for each of the diet treatments in this study were 83 days for *ad libitum*, more than 165 days for 70% caloric restriction, 135 days for 60% caloric restriction and 136 days for late-onset caloric restriction We found that both constant and late-onset calorie restriction increased longevity in grasshoppers by at least 66% than the *ad libitum* group (6).

Since late-onset calorie restriction can rapidly reduce mortality rate the next step is to determine what are the physiological changes involved. One of the leading theories on how calorie restriction extends lifespan is the nutrient allocation hypothesis. The theory holds that when on limiting nutrients, organisms preferentially allocate energy to storage instead of potential offspring (reproduction) (7). This hypothesis made more sense when it was thought that calorie restriction only works if applied long-term. Now that it is known that late-onset calorie restriction quickly lowers mortality rates in flies, late-onset calorie restriction can be used to test whether the rapid effects of calorie restriction are acting through nutrient allocation (8, 9).

We know that a caloric restriction diet, both constant and late-onset, can increase lifespan in lubber grasshoppers. We also know that late-onset can reverse the effects of aging and do so quickly (within 48 h) in flies. However little is known of the physiological responses to calorie restriction. We predict that animals on calorie restriction will allocate resources to self-maintenance instead of growth or reproduction.

Method

<u>Experimental Animals and Rearing:</u>

We used *Romalea microptera* from a colony from Illinois State University. These grasshoppers originated from Miami, Florida, USA. They were kept *en masse* under heat lamps with a 12L:12D photoperiod and a room temperature of 20±2° until they reached adulthood. They were fed Romaine lettuce *ad libitum*, and green beans, oats and green onions occasionally. Only the females were used in the study, since in previous studies it was unclear whether or not caloric restriction enhanced longevity in males (6). When females molted to adulthood they were transferred to a 500 ml ventilated plastic container in order to regulate the amount of food they ate and to ensure competition was not occurring between them. They were also placed in a 14L:10D photoperiod with a corresponding 32°C:24°C thermo cycle.

<u>Treatment Groups:</u>

We tested three diet regimens and a heat shocked group for total fat body protein. Heat shock was used as a positive control since it is an extreme and rapid stress inducer known to rapidly reduce overall protein. As females molted to adult they were serially assigned to four treatments. The diet treatments were: *ad libitum*, starved, and caloric restriction. All diet types were fed *ad libitum* from adult molt until the initiation of treatment at day 20 or 50. Then 48 h before fat body removal they were switched to their diet treatments. The *ad libitum* group was continued on free access to lettuce. The starved group was offered water but no food, and the caloric restriction group was offered 1.5 g of lettuce per day, which was the average amount offered to the 60% caloric restriction group in our previous study. Grasshoppers in the heat shock treatment were fed *ad libitum* during these last 48 h and were heat shocked for at 48°C for 60 min immediately before fat body removal. The mean ± SE number of grasshoppers in each group of the diet treatment groups was 7.67 ±0.21. The number of grasshoppers in the heat shock group was 4 with no variation.

<u>Fat Body Samples:</u>

Fat body samples were collected by decapitating the grasshopper, making an incision along the ventral abdomen and using forceps to remove the entire visceral fat body. Samples were stored in Jerk-lysis buffer (5 ml 1 M Tris HCl, 3 ml 5 M NaCl, 10 ml glycerol, 500µl Triton X-100 and 81 ml dH_2O) at -20°C until analysis. The amount of protein in each sample was determined using a Bradford (1976) assay, with bovine serum albumin standards. Using the absorption (at 630 nm) and the total volume of fat body homogenate, we calculated the amount of protein in each fat body sample.

These data are best interpreted as immediate protein production by fat body, or how much protein the animal was making at the time of decapitation. The majority of protein produced by the fat body of lubber grasshoppers is storage protein, which is then stored in the hemolymph until molting or reproduction (9, 10). Total fat body protein is not a good measure of the individual's current protein storage, but instead estimates future storage. Proteins are important to study in grasshoppers because it is well known that they are the limiting nutrient in grasshoppers.

Statistical analysis:

The data was analyzed by a 2-way ANOVA, with treatment group and day of dissection as factors. Data were log transformed to better meet the assumptions of the test treatments. Also, post-tests (Ryan-Einot-Gabriel-Welsch Multiple Range test) were performed to determine which specific treatments differed. All tests were performed with SAS 9.0 (11).

Results

Treatments strongly effected the total amount of fat body protein ($F_{7,53} = 6.53$; $P < 0.0001$). Both age ($F_{1,7} = 20.6$; $P < 0.0001$) and diet or temperature ($F_{3,7} = 6.06$; $P = 0.0014$) strongly affected total fat body protein. The interaction of age and diet or temperature did not affect total fat body protein ($F_{3,7} = 0.59$; $P = 0.624$). Post-tests (Ryan-Einot-Gabriel-Welsch Multiple Range test) indicated that the day 20 group had greater fat body protein than the day 50 group. Post-tests also indicated that the *ad libitum* group had greater fat body protein than all three other diet or temperature treatments.

Because we had previously tested the effects of calorie restriction starting at day 50 on longevity, this time point was of particular importance. Therefore, we used an ANOVA to directly test whether the *ad libitum* group had greater fat body protein than the other, to avoid a Type II error. When this time point was isolated, the *ad libitum* group did not have greater fat body protein than other groups ($F_{3,25} = 2.00$; $P = 0.143$). Hence, the result of greater fat body protein in the *ad libitum* group than in other groups is largely due to day 20.

Discussion

Studying the effects of diet longevity has benefits. Better understanding of basic physiological processes can lead to new drugs or new ways to heal people. This research can lead to a better understanding of the mechanisms of how the body works. It could also lead to an increase of human longevity and quality of life.

The nutrient allocation hypothesis says that organisms when limited on food will put more energy towards storage instead of offspring (8, 9). The data in the present paper at day 20 supports this hypothesis. At day 20, a female lubber grasshopper is actively producing vitellogenin and yoking oocytes. The *ad libitum* group had a higher amount of fat body protein than the other three treatment groups. This suggests that the other three groups were putting their protein (or energy) into surviving rather than into reproduction. In contrast, by day 50 in a female grasshopper's lifespan they have laid a few of clutches and are ready to die soon, which means there is no need to store so much protein. The fact that less energy (protein) is needed because reproduction is slowing down or has stopped may explain why there is no significant difference between *ad libitum* and the other three treatments protein amounts at this day.

The results obtained from day 20 are a positive control. These results, that protein levels were higher in *ad libitum* than other treatment groups for that day, provide evidence that the diet treatments were effective. Similarly heat shock was used as a positive control because it is known to abruptly lower protein levels in the fat body. Since these positive control treatments were successful, we know that the dietary result from day 50 (i.e., no significant difference between treatments) is a valid.

Previous studies have found that heat shocking mildly increases longevity in flies (14), and our own studies have found that caloric restriction affects longevity (6) and that extreme caloric restriction affects the amount of protein stored in the hemolymph (14). With the knowledge we gained from this experiment we will test the effects of diet on the level of heat shock protein (HSP70) to determine if this protein could be involved in the rapid reduction of mortality seen upon late-onset caloric restriction.

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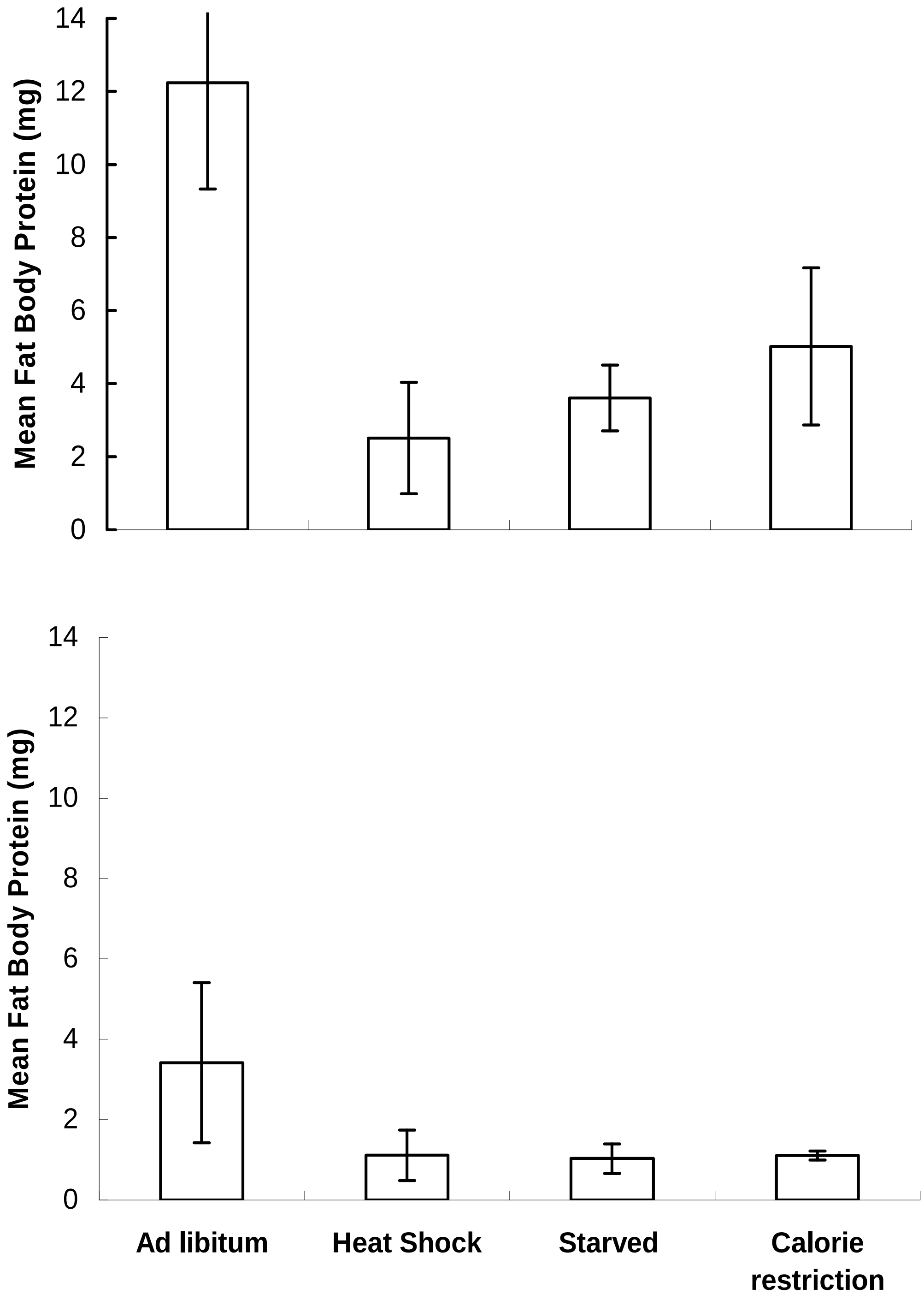
Figure Caption

Figure 1. Mean total protein (mg) in the fat body of each treatment group at day 20 (top) and day 50 (bottom). All groups were fed *ad libitum* on Romaine lettuce prior to the 48 h feeding treatment. These are protein amounts after 48 h of the indicated diets. Starved were only offered water, and caloric restriction were offered 1.5g of Romaine lettuce per day. Heat shock were offered *ad libitum* and heat shocked for 60 min @ 48°C.


Mean Fat Body Protein (mg)
14
12
10
8
6
4
2
0
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14
12
10
8
6
4
2
0
Ad libitum
Heat Shock
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Calorie restriction